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13. ABSTRACT *(Maximum 200 words)*

The adenoviral vector expressing the gene for human B7-1 (Ad.hB7) was prepared. Experiments testing its toxicity in mice began and toxicity studies in rhesus monkeys are planned for next year. Significant improvements were made in culture methods for human breast cancer cells from patient samples. The new method utilizes RT-PCR to assess growth factor receptor expression in breast cancer cells thereby permitting the culture media to be individually tailored. Human breast cancer cell lines and fresh patient samples could both be efficiently transduced with the Ad.hB7 expression vector and the B7 protein remained expressed on the cell surface for over one month. A murine mammary adenocarcinoma model was established and characterized from MT-7 derived from a Balb/c mouse. The growth of this weakly immunogenic tumor was significantly reduced when the tumor cells were transfected to express B7-1. These results demonstrate that the use of human breast cancer cells for gene therapy studies is feasible, that Ad.hB7 efficiently transduces these breast cancer cells with a high percentage of cells expressing B7 long enough to be used as a vaccine, and finally, that the scientific rationale for this approach is sound as shown in an animal model of breast cancer.

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John W. Smith, M.D. 10/22/95

PI - Signature Date

Annual Report For Grant Number DAMD17-94-J-4385
Project Title: "Gene Therapy For Human Breast Cancer"

Table of Contents

Introduction

Nature of the problem

Despite advances in screening, diagnosis, and treatment, breast cancer mortality has remained relatively unchanged. New modalities of treatment are clearly needed to improve the treatment of patients with metastatic disease.

Background

Over the last ten years, treatments designed to harness the enormous power of the immune system to treat cancer have been developed and in some malignancies, have demonstrated significant clinical benefit.

Recent developments have enhanced the understanding of the way T cells recognize tumor associated antigens and the necessity for costimulatory molecules to fully activate T cells. Studies in murine models of cancer have demonstrated that the expression of murine B7 on tumor cells results in tumor rejection of both B7-transfected and wild type tumor cells and that established tumors can be abolished as well.

Purpose of the present work

The hypothesis of this project is that the immunogenicity of human breast cancer cell antigens can be enhanced to therapeutic benefit by the ectopic expression of the T cell co-stimulatory molecule, B7, in these cells. The goal of this project is to test this hypothesis clinically, and the laboratory programs are targeted at the major obstacles to bringing this approach to clinical trial.

Methods of approach

Prior to the initiation of the clinical trial, methods will be developed to isolate highly purified populations of human breast cancer cells from tissue specimens obtained from patients with advanced breast cancer. Experiments will then be conducted to optimize the efficiency and longevity of B7 expression in human breast cancer cells transduced with adenoviral vectors containing the human B7 cDNA. Then, a phase I clinical trial will be conducted testing the safety and immunologic effects of autologous B7 transfected breast cancer cells as a vaccine to create an antitumor immune response in patients with advanced, refractory breast cancer.

Body

The specific aims of this project are dependent on the development of a safe and effective adenoviral vector to efficiently transduce human breast cancer cells. Our collaborators (Drs. Eck and Wilson) at the University of Pennsylvania have made significant progress towards this end during the last year.

Development and toxicity studies of Adenovirus B7 (Ad,hB7)

Based on studies showing that adenovirus vectors efficiently deliver and express genes in primary human breast cancer cells, this laboratory program seeks to develop a certified, replication-defective, recombinant adenovirus bearing a human B7 cDNA (Ad.hB7) for use in human clinical trials.

Vector Development

Ad.mB7 (officially designated H5.020CMVmB7) was constructed through homologous recombination in 293 cells of the large fragment of Cla I digested Ad5 d17001 with a Nhe I linearized plasmid containing adenovirus sequences, the cytomegalovirus immediate early enhancer/promoter element, and the murine B7-1 cDNA. Viral plaques were isolated and screened by flow cytometry and DNA restriction analysis. After several rounds of plaque purification, the virus was prepared in large quantity for animal experiments. Using the same strategy, an adenovirus expressing human B7-1, Ad.hB7, was prepared (officially designated H5.020CMVhB7).

Vector Efficacy Testing

We were aware of the possibility that co-expressed adenoviral proteins might inhibit the immune response to endogenous tumor antigens. Preliminary data using H5.010CBlacZ (expresses lacZ) infected SCK murine mammary carcinoma cells that were retrovirally transduced to stably express murine B7-1 (SCKmB7) indicate that the co-expressed adenovirus proteins do not inhibit the efficacy of B7-1 *in vivo* and may possibly enhance the immune response to endogenous tumor antigens. Experiments to test the ability of H5.020CMVmB7 to mediate tumor rejection in mice are in progress.

Vector Toxicity Testing in the Mouse

We are conducting experiments to determine the best strategy to determine the toxicity of adenovirus-delivered B7-1. Cutaneous and systemic administration of the two adenoviral vectors (mB7 and hB7) will be compared. This will establish whether the vector containing the human cDNA can be adequately tested in mice. If not, then rodent toxicity will be done with the murine B7-1 vector. In either event, the human construct will be tested in nonhuman primates (rhesus monkeys) beginning early next year following its preparation under GMP conditions.

Isolation and purification of human breast cancer cells

The proposed strategy for human breast cancer immunotherapy depends on the isolation of cancer cells from patient specimens and their maintenance in short-term culture for gene transfer. Task 1 in the Statement of Work is to develop methods to isolate highly purified populations of breast cancer cells from tissue specimens of locally recurrent or metastatic sites obtained from patients with advanced, refractory breast cancer. Dr. Steve Ethier who heads this aspect of the project has made significant progress in completing this task.

Over the past year, we have worked to improve our abilities to isolate and culture human breast cancer cells from patient samples. Experiments aimed at improving methods for isolation and culture of human breast cancer cells from patient samples are important in order to achieve efficient transduction of breast cancer cells soon after they are removed from the patient and to provide an opportunity for the preparation of multiple autologous tumor cell vaccines from an individual patient's breast cancer cells. During the past year, we have developed new culture media and new methods for cell isolation that have improved our abilities to culture breast cancer cells for extended periods. These culture media are based on the use of reverse-transcription polymerase chain reaction experiments to assess growth factor receptor expression in breast cancer cells before they are seeded into culture. This is allowing the use of culture media more specifically tailored to an individual patient's breast cancer cells. In particular, we are focusing on that subset of human breast cancers that express the epidermal growth factor (EGF) receptor and are, therefore, dependent on exogenous EGF for growth under serum-free conditions *in vitro*.

During the past year, we have isolated four new human breast cancer cell lines, bringing the total number of cell lines isolated in our laboratory to seven. The establishment of these lines is a direct result of improvements that have been made in the handling of the breast cancer specimens and in the culture media employed. In addition to these established cell lines, we currently have five other breast cancer cell strains that have been maintained in culture for over one month. At present it is not known which if any of these cultures will give rise to still more immortalized cell lines. However, the ability to maintain these cultures for over a month is more than sufficient to allow gene transfer to be carried out using adenoviral expression vectors. Thus, we are currently able to maintain in a viable state, human breast cancer cells from the majority of breast cancer specimens obtained, for periods of time sufficient for the gene transfer protocols to be carried out.

Optimization of B7 expression in Breast Cancer Cells

Task 2 in the Statement of Work is to study and optimize the efficiency and longevity of B7 expression in breast cancer cells transduced with the adenoviral vector containing the human B7 cDNA (adeno-B7 vector). Over the past year, we have focused on the initial testing of the adeno-B7 expression vector in our established human breast cancer cell lines as well as in freshly isolated human breast cancer cells. These experiments have established that human breast cancers from different patients can be efficiently transduced with this vector. Multiplicities of infection (MOI) of between 10^3 and 10^4 per cell are required, depending on the individual cell line or strain. For example, infection of SUM-52PE cells with the adeno-B7 expression vector at a multiplicity of infection of 1,000 results in greater than 50% of the cells expressing B7-1 at 48 hours after infection. We have also shown that once cells become infected, they express the B7 protein on their cell surface for over one month with little change in expression levels. Thus, this expression vector results in B7 gene expression for periods long enough to be able to act as a co-stimulatory molecule.

In more recent experiments, freshly isolated human breast cancer cells were transduced with the adeno-B7 vector. Like the established cell lines, these cells were also successfully infected with this vector and expressed B7 protein on the cell surface. In some cases, higher MOI's were needed with the freshly isolated cells than with some established cell lines. Nevertheless, these preliminary studies have established the feasibility of transducing B7 protein into freshly isolated human breast cancer cells for use as an autologous tumor cell vaccine.

Development of an animal model of gene therapy for breast cancer

In the technical merit evaluation of this grant proposal, the reviewers made the following comments:

> *"There is some question as to whether the phase I clinical trial should proceed as planned or be delayed until a better animal model is developed."*
>
> *"Although the reviewers recognize the expertise of the PI and associates, it is suggested that the laboratory and animal studies be completed before initiating clinical trials."*

Because of these suggestions, Dr. Alfred Chang's laboratory has conducted a series of experiments over the last year to develop a better animanl model of gene therapy for breast cancer.

Introduction

Investigations were initiated to develop a murine model of mammary adenocarcinoma to evaluate the role of various immunomodulatory peptides (ie, B7-1 and cytokines) in altering the host immune response. Such a model will allow evaluation of these peptides in enhancing the immunogenicity of tumor for the eventual purposes of developing vaccines for immunotherapy of established disease. The specific aims of these studies include: 1) to establish and characterize a murine mammary adenocarcinoma animal model, 2) to transduce and characterize a murine mammary adenocarcinoma line with cDNAs encoding for immunoregulatory peptides and 3) to develop vaccine strategies for the treatment of established metastases utilizing genetically modified tumor lines.

Establish and characterize a murine mammary adenocarcinoma model.

We obtained a mammary adenocarcinoma from co-investigator Dr. Stephen Ethier, which was originally designated MT-7 and derived from the syngeneic Balb/c host (1). A subline of this tumor was obtained by inoculating this tumor subcutaneously in Balb/c mice and isolating a metastatic nodule by re-passage in animals as well as establishment of a culture line. This subline was renamed MT-901 and was found to be reliably tumorigenic upon re-passage *in vivo*. The growth characteristics of MT-901 with the s.c. inoculation of various doses of tumor cells is shown in Figure 1.

The irradiation of these tumor cells with $\geq$ 5,000 cGy will eliminate their tumorigenicity. In Figure 2, various doses of radiation were exposed to MT-901 tumor prior to inoculation of 10^6 cell s.c. There were 5 mice in each group.

MT-901 is a weakly immunogenic tumor. Mice were inoculated s.c. with 10^6 cells that had been irradiated with 5,000 cGy which was previously established to be non-tumorigenic in 5 mice. Two weeks later, 10^6 viable MT-901 cells were inoculated as a tumor challenge in these animals and compared to a control group of animals (n = 5) that had not received irradiated cells (Figure 3). The rate of tumor growth was diminished in the animals that had received prior immunization with irradiated tumor cells.

Our laboratory has previously described the phenomenon of pre-effector cell induction in lymph nodes (LN) draining sites of progressive tumor (2, 3). LN cells derived from hosts bearing s.c. inoculated tumor will harbor cells which mediate antitumor reactivity after secondary *in vitro* activation with anti-CD3 monoclonal ab (anti-CD3) for 2 days and subsequent expansion in IL-2 (10 u/ml) for 3 days. We proceeded to evaluate the

antitumor reactivity of MT-901 tumor-draining lymph node (TDLN) cells. The Renca tumor, which is a renal cell carcinoma line syngeneic to the Balb/c host, was used as a specificity control. MT-901 cells were inoculated s.c. (10^6 cells) into the flanks of animals, and TDLN were harvested 9 to 10 days later for anti-CD3/IL-2 activation. After activation, the cells were evaluated for antitumor reactivity by measurement of cytokine released into the supernatant (ie., GM-CSF and INFγ) after a 24h exposure to irradiated tumor cells *in vitro*. As shown in Figure 4, activated MT-901 TDLN cells specifically released GM-CSF and IFNγ into the culture supernatant upon exposure to MT-901 tumor and not the unrelated Renca tumor indicating that specific immunologic recognition had been induced.

In other tumor models, we have reported that the tumor-specific release of GM-CSF and IFNγ are excellent indicators that immune cells will have the functional capacity to mediate tumor regression in adoptive immunotherapy (4). To evaluate this, we examined whether activated MT-901 TDLN were capable of mediating tumor regression in an adoptive immunotherapy model. Groups of mice (n = 5/group) were inoculated i.v. to establish MT-901 or Renca pulmonary metastases. Three days later, animals were inoculated with 6 x 10^6 activated MT-901 TDLN cells i.v. Some groups of animals were also administered IL-2 (5,000 units i.p. bid x 4 days). Animals were sacrificed approximately 21 days after tumor inoculation and the pulmonary metastases enumerated in a blinded fashion (Table 1). Animals with MT-901 pulmonary metastases had significant regression of tumor when treated with MT-901 TDLN and IL-2. This response was immunologically specific since Renca pulmonary tumors were not affected.

Table 1: MT-901 TDLN Cells Mediate the Specific Regression of Pulmonary Metastases

	MT-901 TDLN[a]	IL-2	Lung Tumor	Mean No. of Lung Metastases (SEM)
A.	--	--	MT-901	247 (5)
B.	--	+	MT-901	205 (51)
C.	+	+	MT-901	2 (2)[a]
D.	--	--	Renca	249 (3)
E.	--	+	Renca	175 (92)
F.	+	+	Renca	275 (35)

[a] $P < 0.001$ compared to A and B groups.

Transduce MT-901 with cDNAs encoding for immunoregulatory peptides

Recently, several laboratories have reported that tumors can demonstrate significant alterations of host immune responsiveness if they are genetically modified to express certain immunoregulatory peptides. Towards that objective, we evaluated the genetic modification of MT-901 with two immunoregulatory peptides: B7-1 and GM-CSF. B7-1 is a co-stimulatory molecule which binds to the CD28 receptor expressed on T cells and is involved in cellular activation events. The presentation of tumor antigens in the absence of this co-stimulatory signal may lead to tolerance and may explain why many tumors are not rejected by the host. GM-CSF is a potent stimulator of antigen presenting cells (APC) such as dendritic cells or macrophages.

Using a physical method involving B7-1 cDNA plasmid attached to dendrimers, the MT-901 was transfected to express B7-1. The wild-type tumor does not normally express this molecule (Figure 5). A clone of a transfectant, designated MT9B14 was isolated because of its high expression of B7 (Figure 5).

The tumorigenicity of MT9B14 was assessed by inoculating 10^6 cells s.c. into mice and compared with a group of control mice that received wild-type tumor (n = 5). In Figure 6, one can see that 4 of 5 mice demonstrated reduced tumorigenicity compared with control animals including one animal where no growth occured and another where initial growth was followed by tumor shrinkage to the point of no detectable growth.

Utilizing a retroviral vector containing the MFG backbone, a gene encoding murine GM-CSF was transduced into MT901. Several clones were isolated which secreted GM-CSF and the highest secreting one (MT9G1) was selected for further study. MT9G1 was found to secrete approximately 240 ng/10^6 cells/24h of GM-CSF as measured by ELISA. The tumorigenicity of MT9G1 was assessed by inoculating 10^6 cells s.c. into mice and compared to animals receiving wild-type tumor. As shown in Figure 7, MT9G1 was not significantly altered with respect to its *in vivo* growth characteristics.

Develop vaccine strategies for the treatment of established metastases utilizing genetically modified tumor cells.

Based upon our previous work involved with adoptive T cell immunotherapy, we investigated the ability of the derived MT-901 transfectants to elicit immune cells in TDLN. In 2 separate experiments, 10^6 MT-901, MT9B14 and MT9G1 were inoculated s.c. in the flanks of animals and TDLN harvested 10 days later for anti-CD3/IL-2 activation. After activation, 2 x 10^7 TDLN cells were adoptively transferred i.v. into animals with 3-day established MT-901 lung metastases. Some groups of mice received IL-2 (5,000 units i.p. bid x 4 days) commencing on the day of cell transfer. After 14 days from i.v. tumor inoculation, lungs were harvested and tumor nodules enumerated (n = 5 mice/group). The results are summarized in Table 2.

Table 2: Therapeutic Efficacy of TDLN Derived from MT-901 Transfectant Tumors

			Mean No. of Lung Metastases (SEM)	
	Effector Cells	IL-2	Exp. 1	Exp 2
A.	--	--	>250	>250
B.	--	+	206 (43)	210 (39)
C.	MT-901 TDLN	+	123 (42)[a]	85 (33)[a]
D.	MT9B14 TDLN	+	56 (26)[b]	197 (41)
E.	MT9G1 TDLN	+	16 (4)[c]	37 (18)[c]

[a] $P < 0.05$ compared to groups A-B.

[b] $P < .05$ compared to groups A-C.

[c] $P < .05$ compared to groups A-D.

These studies demonstrated that GM-CSF secretion by MT9G1 was superior to MT-901 and MT9B14 in the induction of immune cells in TDLN in both experiments. Expression of B7-1 was inconsistent in enhancing sensitization of TDLN compared with wild-type tumor and was observed in only 1 of the 2 experiments. In a survival experiment, MT9G1 TDLN cells were superior to MT901 TDLN in prolonging the survival of animals treated with macroscopic day-10 lung metastases (data not shown).

In other studies, we have found that activated MT9G1 TDLN cells release greater amounts of IFNγ in response to *in vitro* stimulation with wild-type tumor compared to MT-901 TDLN cells (data not shown). The results of these studies have been accepted for presentation at the Fourth International Conference on Gene Therapy of Cancer to be held November 9-11, 1995 in San Diego, California (4).

Conclusions

During the first year of funding for this grant several milestones have been achieved and we are able to make the folowing conclusions:

1. The adenoviral expression vector for the human B7-1 gene was successfully prepared.

2. Studies demonstrated that the co-expressed adenoviral proteins did not inhibit the efficacy of B7-1 *in vivo* in a murine model.

3. Toxicity studies started in mice and will begin next year in rhesus monkeys.

4. Significant improvements were made in culture methods for human breast cancer cells from patient samples. The new method utilizes RT-PCR to assess growth factor receptor expression in breast cancer cells thereby permitting the culture media to be individually tailored.

5. Human breast cancer cell lines and fresh patient samples could both be efficiently transduced with the Ad.hB7 expression vector with a high percentage of the cells expressing the B7 protein and this protein remained expressed on the cell surface for over one month.

6. These results demonstrate that the use of human breast cancer cells for gene therapy studies is feasible and practical. The demonstration that the Ad.hB7 expression vector efficiently transduces these breast cancer cells with a high percentage of cells expressing B7 for over a month indicates that these cells can be used to vaccinate patients in an attempt to induce T cell immunity against their autologous tumor.

7. A murine mammary adenocarcinoma model was successfully established from MT-7 in the Balb/c mouse and renamed MT-901. MT-901 is a highly tumorigenic murine mammary tumor line that is only weakly immunogenic.

8. Transfectant clones of MT-901 that were generated to express B7-1 demonstrated reduced tumorigenicity compared to MT-901 supporting the scientific premise of the proposed clinical trial, i.e. that the expression of B7-1 on the surface of autologous cancer cells will induce an immune response capable of altering the growth of the tumor.

9. GM-CSF secretion by an MT-901 transfected clone is superior to MT-901 and a B7-1 expressing clone in the sensitization of TDLN cells utilized in an adoptive immunotherapy model. This animal model has therefore demonstrated a potential limitation of the vaccine approach using B7-1 transduced tumor cells and will serve as an important animal model to test additional ideas to enhance the vaccine strategy to be used in breast cancer. In addition to the use of GM-CSF secreting tumor cells, future studies will evaluate the immunomodulatory role of IL-12 with or without B7-1 expression in this model.

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1. Margaretten NC, Witschi H: Effects of hyperoxia on growth characteristics of metastatic murine tumors in the lung. Cancer Res. 48:2779-2783, 1988.

2. Yoshizawa H, Chang AE, Shu S: Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. J. Immunol. 147:729-737, 1991.

3. Geiger JD, Wagner PD, Cameron MJ, Shu S, Chang AE: Generation of T cells reactive to the poorly immunogenic B16-BL6 melanoma with efficacy in the treatment of spontaneous metastases. J. Immunother. 13:153-165, 1993.

4. Aruga E, Aruga A, Arca MJ, Smith JW, Chang AE: Immunotherapy of murine breast cancer by lymph node cells primed by tumor modified to secrete GM-CSF. (To be presented at 4th International Conference on Gene Therapy of Cancer), San Diego, CA, November 1995.

Figure 1

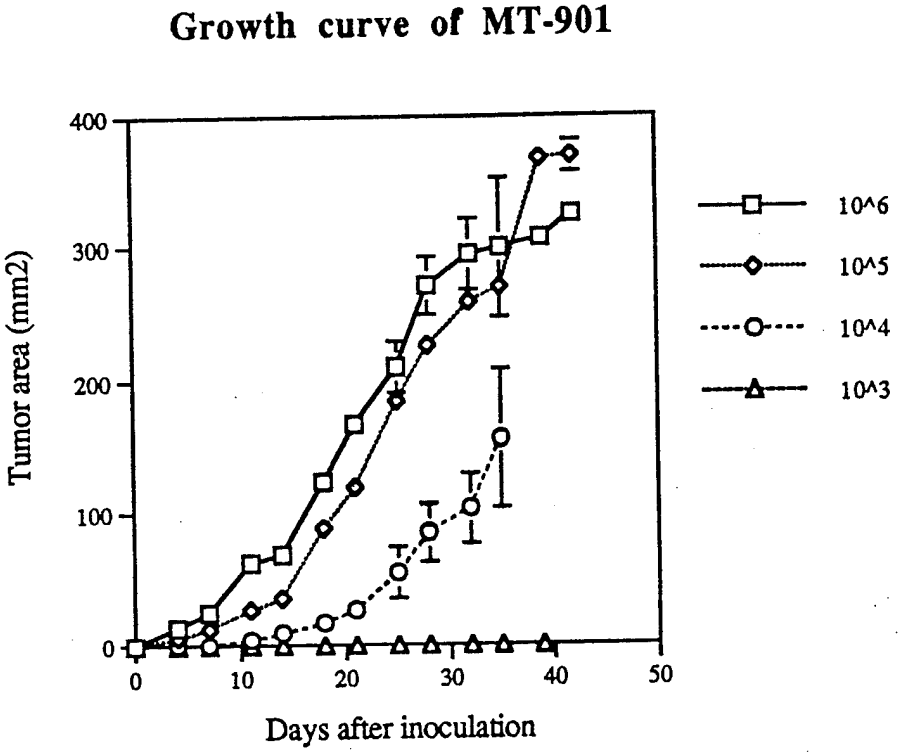
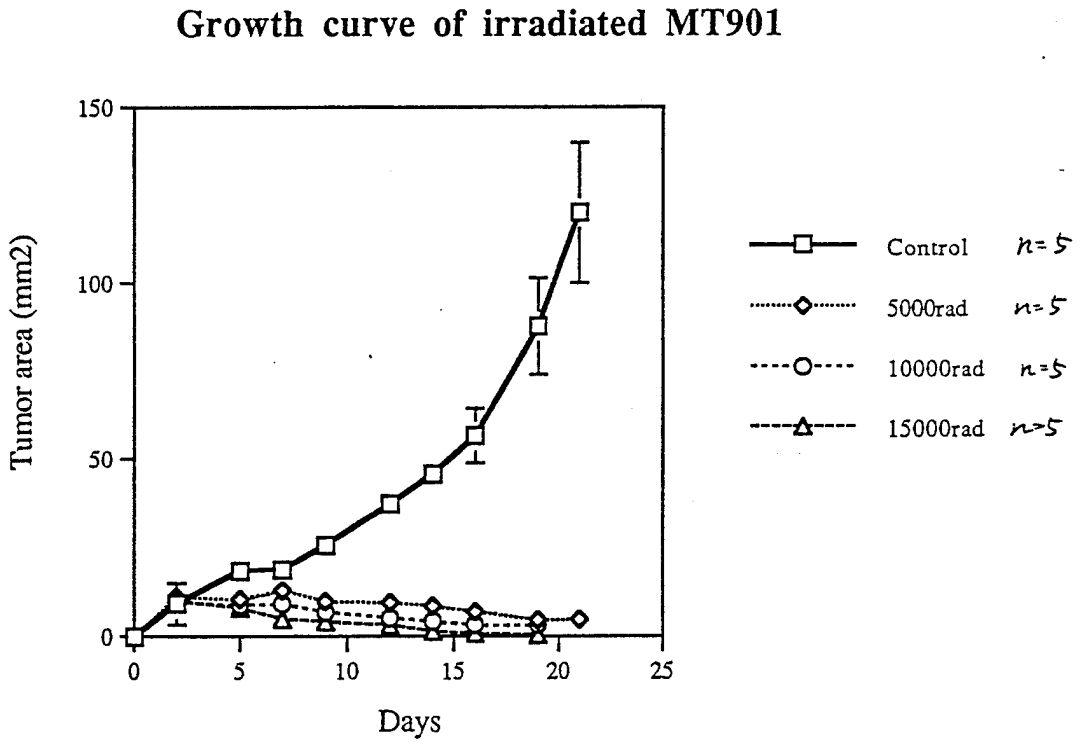

Growth curve of MT-901
Tumor area (mm2)
400
300
200
100
0
0
10
20
30
40
50
Days after inoculation
10^6
10^5
10^4
10^3

Figure 2



10^6 cells injected i.d.

Figure 3

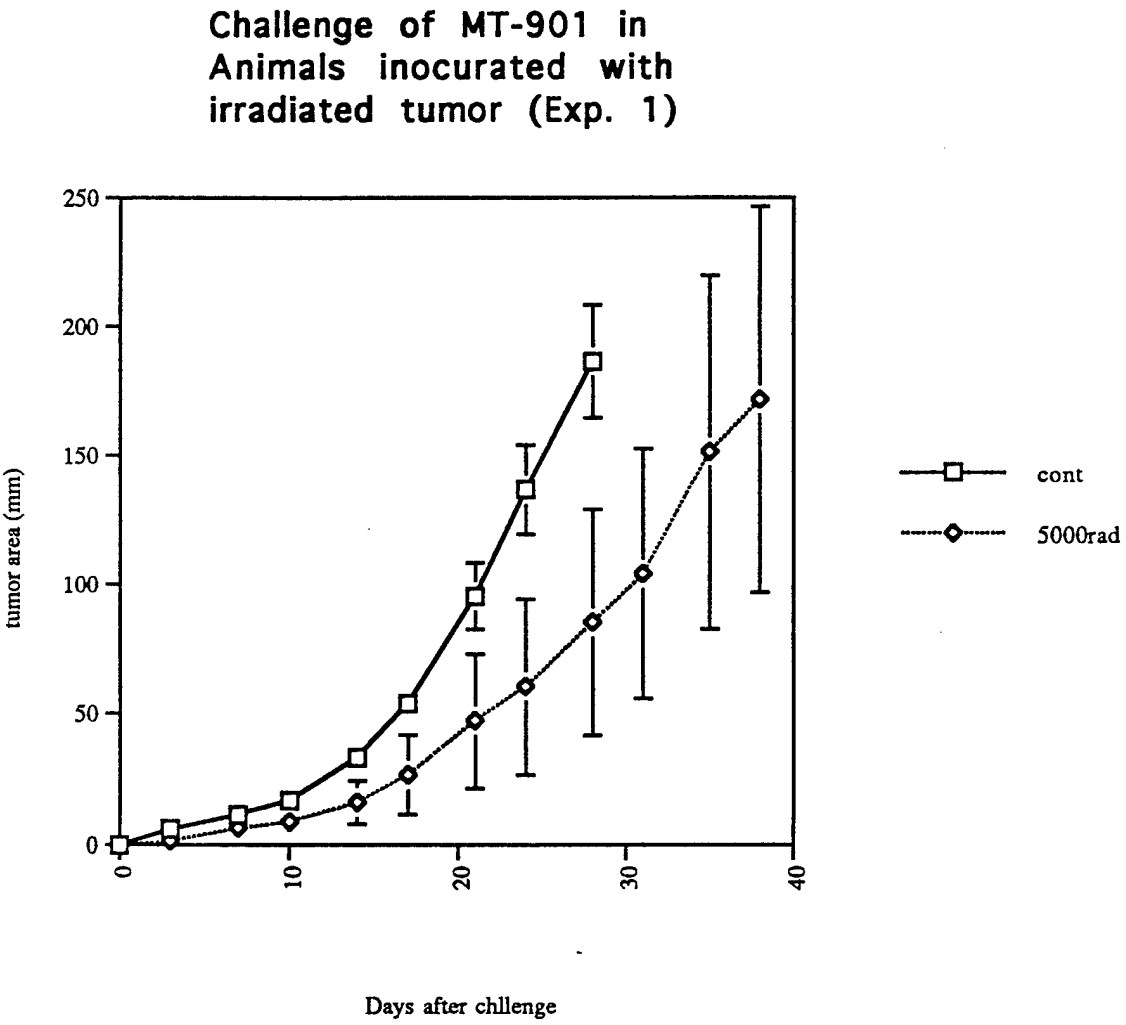
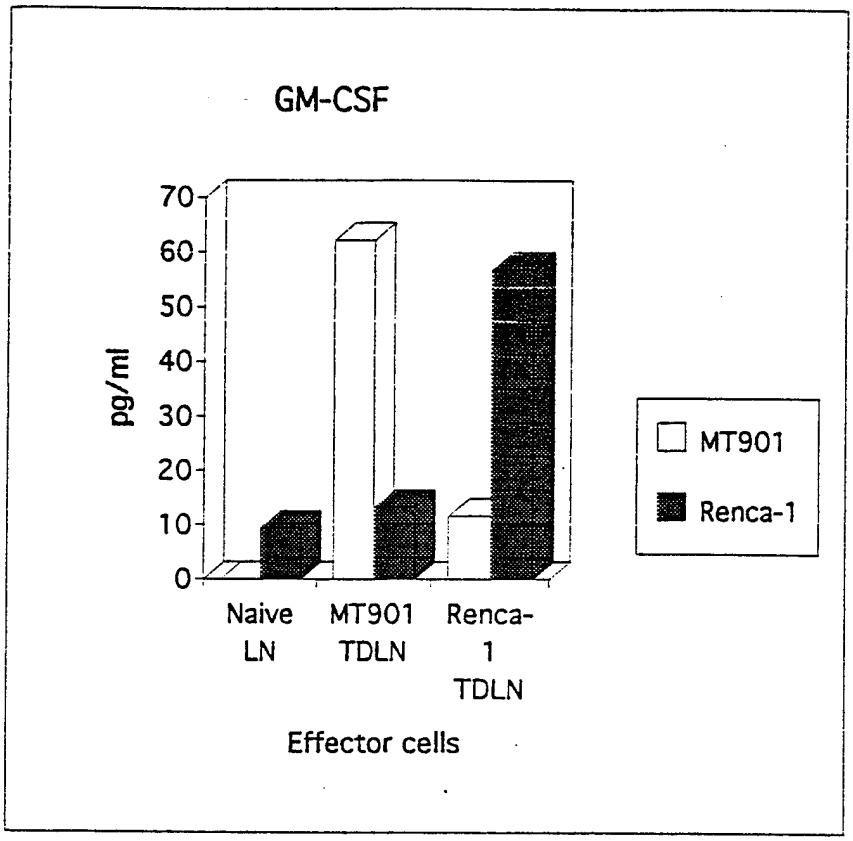

Challenge of MT-901 in Animals inocurated with irradiated tumor (Exp. 1)
tumor area (mm)
250
200
150
100
50
0
0
10
20
30
40
Days after chllenge
cont
5000rad

Figure 4


GM-CSF
pg/ml
70
60
50
40
30
20
10
0
Naive LN
MT901 TDLN
Renca-1 TDLN
Effector cells
MT901
Renca-1

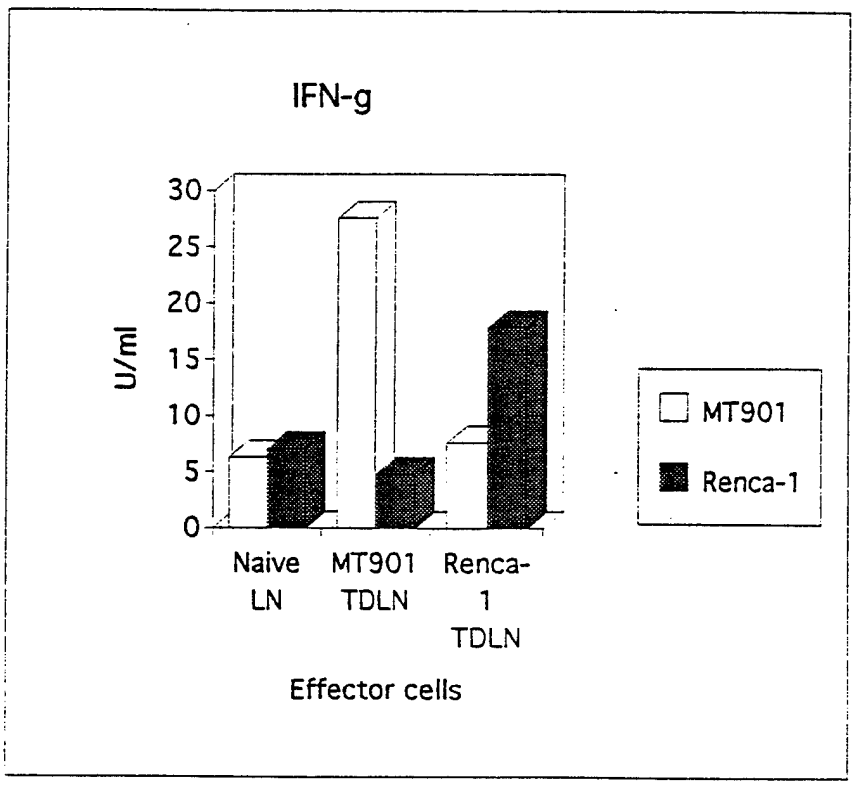
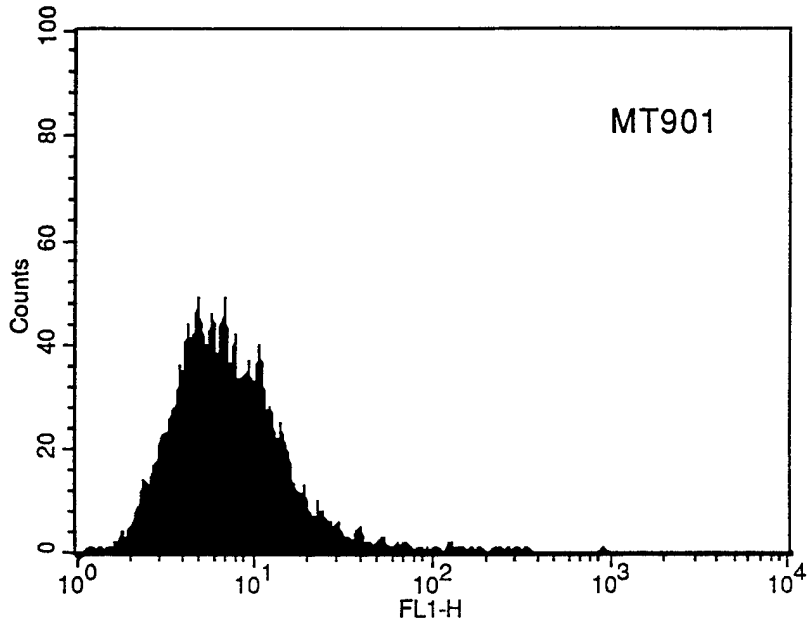
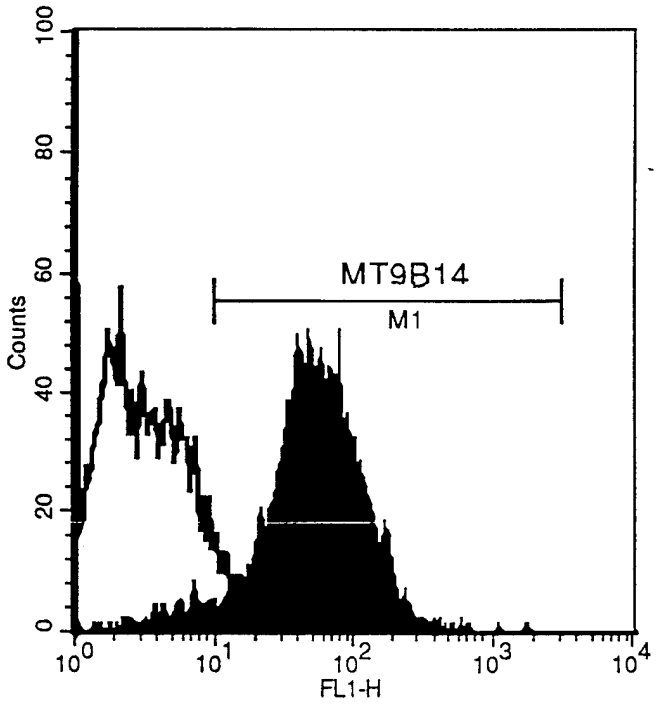

IFN-g
U/ml
30
25
20
15
10
5
0
Naive LN
MT901 TDLN
Renca-1 TDLN
Effector cells
MT901
Renca-1

Figure 5

B7 expression of MT9B14

Wild Type MT901's expression . overlapping





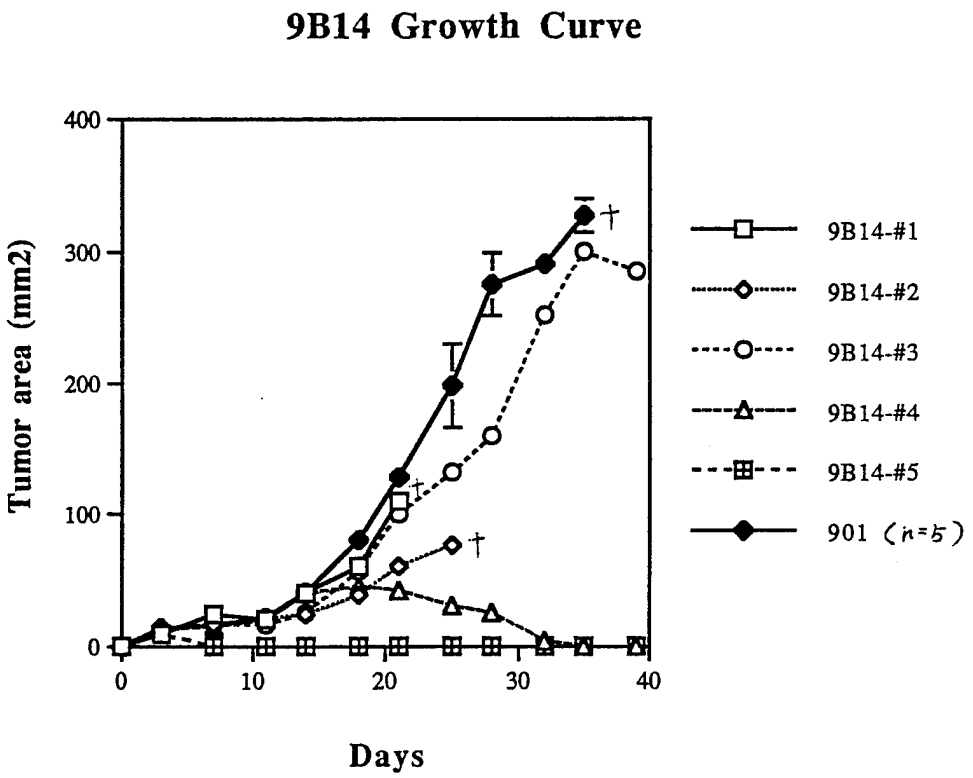
Histogram Statistics

File: Data.030

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	7113	100.00	71.13	64.56
M1	10, 3106	6831	96.04	68.31	66.97

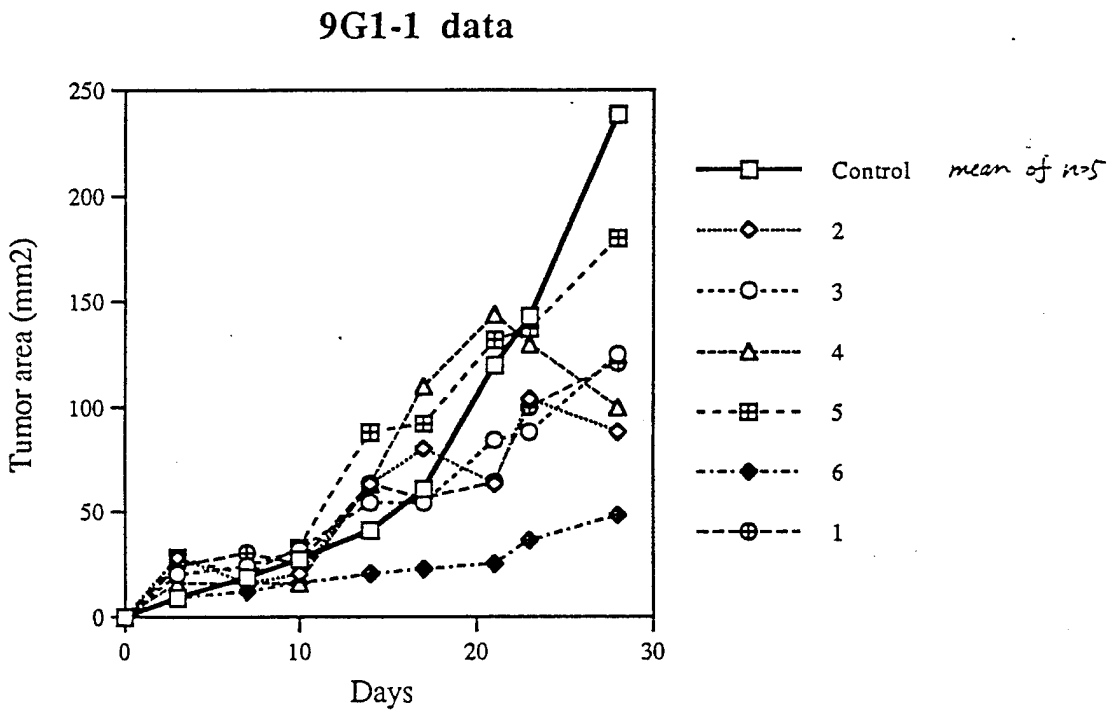
*9B14 : 14th clone of MT901 transfected B7

Figure 6



10^6 cells id. midline of abdomen

Figure 7


9G1-1 data
Tumor area (mm2)
250
200
150
100
50
0
0
10
20
30
Days
Control
mean of n=5
2
3
4
5
6
1